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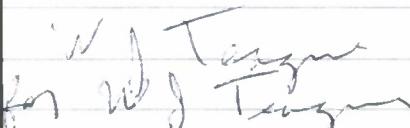
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Technical Note: Influence of Experimental Conditions on the Outcome of Laboratory Investigations Using Natural Coastal Seawaters

J.S. Lee,^{‡,*} R.I. Ray,^{*} and B.J. Little^{**}

ABSTRACT

Methods for handling and storing natural coastal seawater, particularly methods for deaeration over time, influenced the chemistry and microflora. Bubbling nitrogen into natural seawater to achieve an anaerobic condition was not conducive to the growth of sulfate-reducing bacteria (SRB). Despite a higher initial dissolved sulfate concentration and higher total organic carbon in Persian Gulf seawater, higher dissolved sulfide concentrations and SRB populations were measured in Key West, Florida, seawater under all exposure conditions.

KEY WORDS: carbon steel, marine corrosion, seawater, sulfate-reducing bacteria

INTRODUCTION

Sulfate-reducing bacteria (SRB) were the first group of microorganisms identified as causing corrosion.¹ SRB have been the focus of many investigations involving microbiologically influenced corrosion (MIC), and several corrosion mechanisms have been attributed to SRB, including cathodic depolarization by the enzyme dehydrogenase, anodic depolarization, production of iron sulfides, release of exopolymers capable of binding metal ions, sulfide-induced stress corrosion cracking, and hydrogen-induced cracking or blistering. In

a 1986 volume of contributed papers, 10 of 36 papers specifically mention SRB in the title.² In the 2009 session on MIC at CORROSION 2009, 3 of 10 papers dealt with some aspect of SRB-influenced corrosion. Today the list of causative and contributing bacteria, fungi, and archaea includes numerous groups;³ however, SRB are arguably the microorganisms most closely identified with MIC.

SRB are a group of ubiquitous, diverse anaerobes that use sulfate (SO_4^{2-}) as the terminal electron acceptor, producing sulfide (S^-). SRB have been isolated from a variety of environments.⁴⁻⁵ Because of the consistently high concentration of sulfate in seawater (>2 g/L), SRB are extremely important in seawater corrosion. For many years there has been a debate over the best laboratory solution for studying marine corrosion,⁶ i.e., natural or artificial seawater. Dexter carefully compared laboratory solutions and natural seawater for studying the corrosion of aluminum alloys and prepared a specific artificial medium that reproduced the short-term behavior of aluminum alloys in natural seawater.⁶ However, none of the laboratory solutions were able to simulate the long-term corrosion behavior of aluminum alloys in natural seawater. The selection of a laboratory medium for MIC experiments is even more complicated. Investigators have used natural seawater with naturally occurring microorganisms,⁷⁻⁹ artificial seawater to which nutrients and microorganisms have been added,¹⁰⁻¹¹ and inoculated synthetic media, e.g., Postgate B with 3.5% sodium chloride (NaCl)¹² and Postgate C¹³ in MIC experiments. The use of the term synthetic sea-

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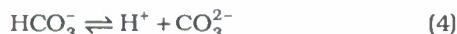
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ter is not always defined. Vargas-Avila and coworkers defined 0.6 M NaCl as synthetic seawater.¹² In other cases, the term synthetic seawater connotes commercially available carbonate-buffered salt solutions that mimic the composition of seawater.

To further complicate interpretation and comparison of data, many laboratory experiments with SRB rely on gas purging to remove oxygen and create an anaerobic environment in the laboratory solution of choice. The pH of carbonate-buffered synthetic and natural seawater is controlled (buffered) by carbon dioxide (CO₂). Gaseous carbon dioxide (CO₂[g]) from the atmosphere dissolves into seawater forming aqueous carbon dioxide (CO₂[aq]) (Equation [1]), reacts with water, and forms carbonic acid (H₂CO₃) (Equation [2]). Proton dissociation from H₂CO₃ proceeds with interdependence on pH. The first proton dissociation forms bicarbonate (HCO₃⁻) (Equation [3]), with carbonate (CO₃²⁻) formation from subsequent proton dissociation (Equation [4]).



Increasing atmospheric [CO₂[g]] increases dissolved [CO₂[aq]], which elevates [H₂CO₃] resulting in decreased seawater pH. If CO₂[aq] is removed from seawater, pH increases. In some cases, researchers using a seawater medium and a gaseous nitrogen (N₂) purge have indicated the need for pH control,¹¹ whereas others¹⁴ have not. Experiments described in this paper were designed to evaluate microbial sulfide production in two natural coastal seawaters collected from Key West, Florida, and the Persian Gulf deaerated under two laboratory conditions: purging with N₂ and maintenance in a hood with a mixture of gases, including N₂, hydrogen (H₂), and CO₂.

MATERIALS AND METHODS

Seawater Collection

Persian Gulf (PG) seawater was collected from the United States Navy pier at Mina Sulman, Bahrain. Key West (KW), Florida, seawater was collected at the Naval Research Laboratory's Marine Corrosion facility. PG seawater was collected within the first meter of the seawater surface by immersing each container underwater by hand. KW seawater was collected at a depth of 1.2 m to 1.5 m under water by intake pipes pow-

ered by suction pumps. Seawater was shipped to the Naval Research Laboratory (NRLSSC), Stennis Space Center, Mississippi, in three 5-gal (18.93 L) plastic containers, each containing 4 gal (15.14 L) of seawater. Both waters had been collected 48 h previous to arrival at NRLSSC and testing began within 24 h of arrival.

Exposure Conditions

Seawater samples from the two locations were maintained in the following conditions in the laboratory:

- PG-A/KW-A: 450 mL stagnant seawater in a loosely covered glass container exposed to air.
- PG-D/KW-D: 1,500 mL stagnant seawater maintained inside an anaerobic hood in a 2,000-mL glass flask sealed with a rubber stopper.
- PG-B/KW-B: 1,500 mL stagnant seawater contained in a 2,000-mL glass flask, bubbled with house-supplied nitrogen gas, and sealed with a rubber stopper.

The atmosphere in the anaerobic chamber⁽¹⁾ was maintained using a gas mixture of 10% H₂, 5% CO₂, and 85% N₂. Hydrogen was added to the anaerobic gas mixture to aid in the removal of O₂. A palladium catalyst inside the anaerobic chamber served as an O₂ and H₂ reaction site resulting in the formation of water and increased humidity. Relative humidity was maintained between 20% and 30% using an alumina desiccant. Atmospheric O₂ concentration was monitored continuously at less than 1 part per million (ppm). House-supplied N₂ gas specifications called for less than 100 ppm atmospheric O₂; however, during the duration of the experiment the average concentration was ~10 ppm O₂. Bubbling was not vigorous with approximately 2 bubbles per second. No nutrients or other additions were made to either of the seawaters.

Seawater Analysis

At the onset of the experiment and continuing at 30-day intervals, 50 mL of seawater was removed from each exposure condition for analysis. The pH was measured using a calibrated pH meter. Dissolved sulfide concentrations were determined in triplicate using the methylene blue method 228-C.¹⁵ Four sterile 3-mL syringes were used to remove 4 mL (1 mL in each syringe) from the 50-mL seawater sample. One mL was used to inoculate serial dilutions (10¹⁰) in each of the following seawater media:⁽²⁾ phenol red dextrose broth, Postgate medium B, nutrient broth, and thioglycollate medium to determine most probable numbers (MPN) of acid-producing bacteria (APB), SRB, general heterotrophic aerobes, and anaerobes, respectively. Dilutions were incubated for 28 days at 25°C. Salinity, total organic carbon, and sulfate were also quantified at the onset of the experiment for both seawaters.⁽³⁾

⁽¹⁾ Coy Laboratory Products, Grass Lake, Michigan.

⁽²⁾ Dixie Testing and Products, Inc., Houston, Texas.

⁽³⁾ AccuLab Inc., Marrero, Louisiana.

CO₂ vs. pH

Gas mixtures were prepared⁽⁴⁾ with CO₂% ranging from 0.01% to 0.1%, 10% H₂, and balanced with N₂. Bottled laboratory-grade N₂ with 0% CO₂ was also used. KW seawater (500 mL) was added to a sealed 1,000-mL flask with an electronic pH meter. Starting with N₂ and 0% CO₂, gas was bubbled into seawater using a pressure regulator set to 1 psi. The pH was allowed to stabilize over a 1-h period, pH was recorded, and the gas mixture with the next highest CO₂% value was introduced.

RESULTS***Seawater Analysis***

Seawater chemistry analyses for both seawaters at the onset of the experiment are listed in Table 1. PG seawater had higher values for all parameters when compared to KW seawater. Table 2 lists the bacterial counts for the four different bacterial groups tested for both seawaters at the onset of the experiment. KW seawater had equal or greater numbers of each group of bacteria when compared to PG seawater.

⁽⁴⁾ Nordan-Smith Specialty Gases, Hattiesburg, Mississippi.

TABLE 1
Seawater Physical Chemistry

Seawaters	pH	Salinity (g/L)	Total Organic Carbon (mg/L)	Sulfate (mg/L)
Key West	7.82	38	1.79	3,864
Persian Gulf	7.98	44	1.94	4,696

The pH values were monitored over the exposure period (Figure 1). There were no significant differences in pH between the two coastal seawaters for the same exposure condition. However, different exposure conditions produced a range of pH values that varied by 3 pH units. Stagnant seawaters exposed to air maintained a pH near 8.0 (PG-A/KW-A). Exposure to the mixed gas anaerobic environment decreased pH values to below 7.0, with KW-D having the lowest recorded value of 6.5. Bubbling N₂ gas had the opposite effect, causing an increase in pH to above 9.0.

Dissolved sulfide concentrations were monitored over time for all exposure conditions (Figure 2). Conditions exposing the sample to air produced dissolved sulfide levels below the resolution (<1 parts per billion [ppb]) of the detection method and are not shown

TABLE 2
Bacterial Cell Counts at Onset of Experiment

Seawaters	Heterotrophic Aerobes (10 ³)	Heterotrophic Anaerobes (10 ³)	Acid- Producing Bacteria (10 ³)	Sulfate- Reducing Bacteria (10 ³)
Key West	6	4	3	2
Persian Gulf	6	2	2	1

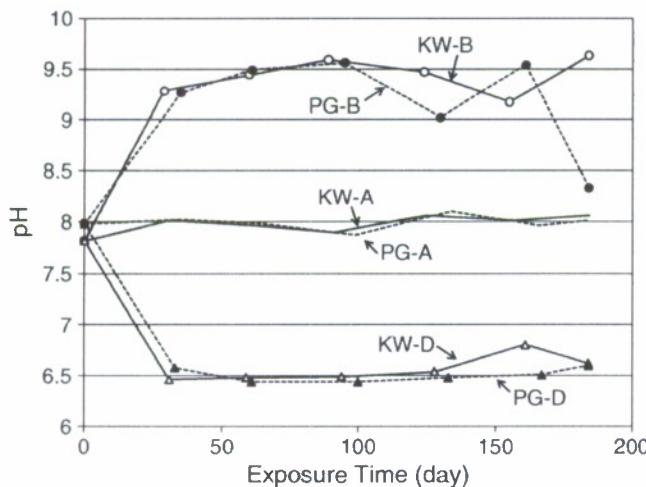


FIGURE 1. pH values as a function of exposure time (days) for Key West seawater (KW-) and Persian Gulf seawater (PG-) under aerobic (A), nitrogen-bubbled (B), and anaerobic (D) conditions.

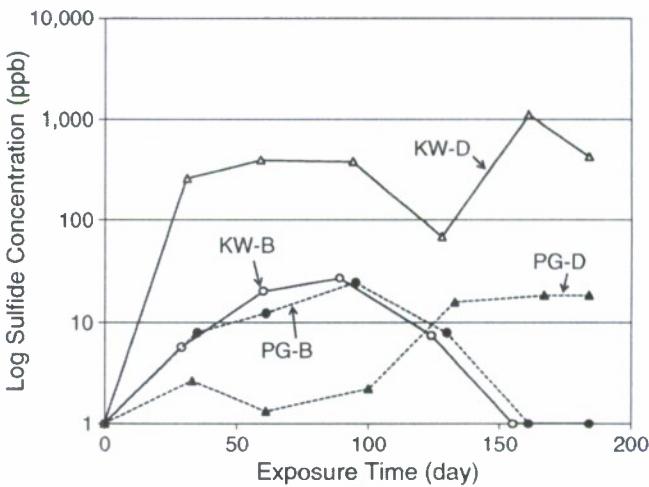


FIGURE 2. Log sulfide concentration in ppb as a function of exposure time (days) for Key West seawater (KW-) and Persian Gulf seawater (PG-) under aerobic (A), nitrogen-bubbled (B), and anaerobic (D) conditions.

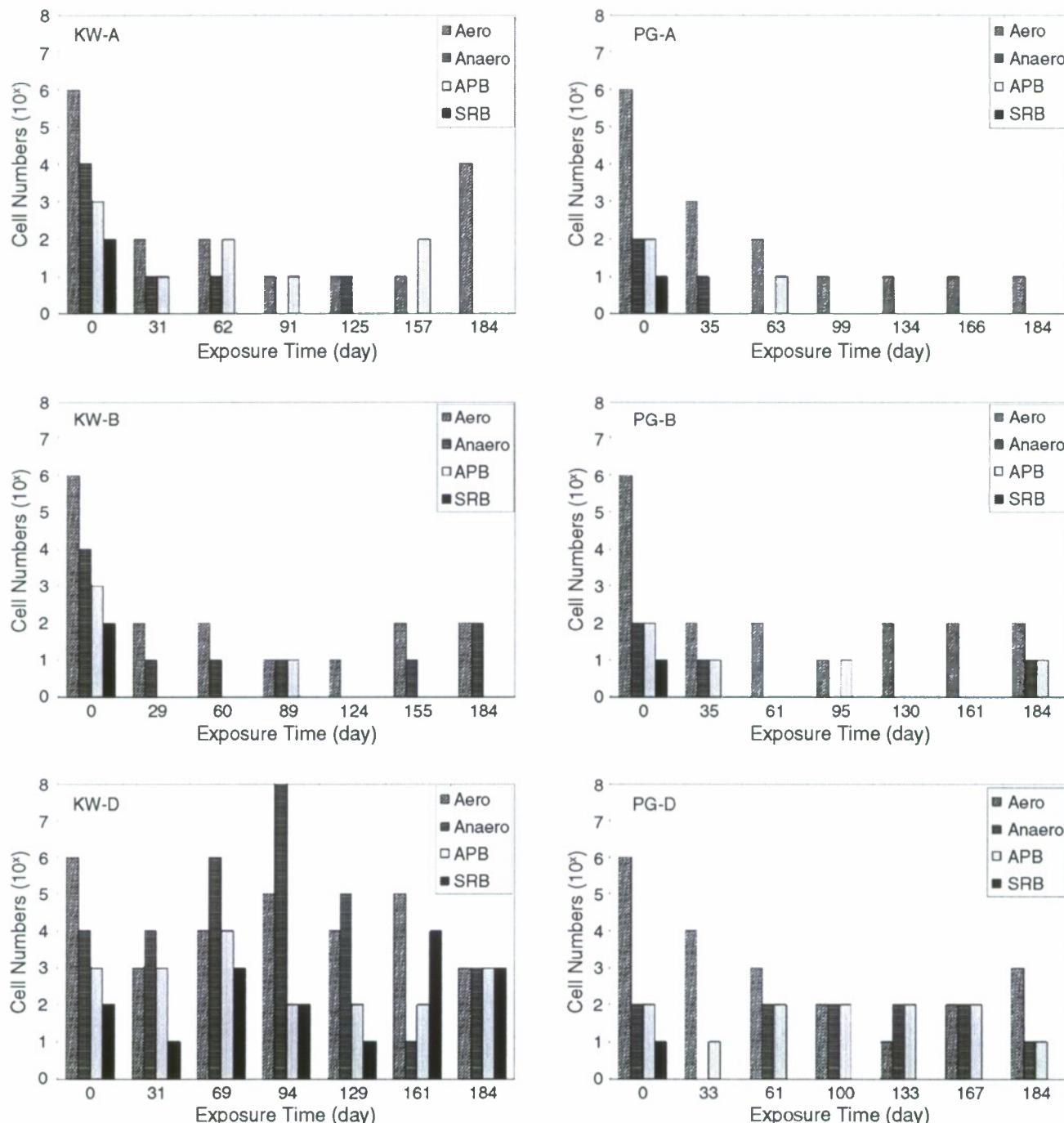


FIGURE 3. Bacterial cell numbers (10^x) as a function of exposure time (days) for Key West seawater (KW-) and Persian Gulf seawater (PG-) under aerobic (A), nitrogen-bubbled (B), and anaerobic (D). Bacterial groups include general heterotrophic aerobes (AERO) and anaerobes (ANAERO), acid-producing bacteria (APB), and sulfate-reducing bacteria (SRB).

in Figure 2. Anaerobic stagnant seawaters maintained in a hood with mixed gases (KW-D/PG-D) produced more than 10^1 ppb to 10^3 ppb dissolved sulfide. Seawaters maintained with bubbled nitrogen produced 10^1 ppm dissolved sulfide during the course of the experiment, but dissolved sulfide was below detection limits at the conclusion of the experiment.

Planktonic bacterial populations were monitored over time for all exposure conditions and the results

are shown in Figure 3. Generally, KW seawater had higher bacterial numbers in all exposure conditions when compared to PG seawater. Bubbling N₂ gas through the seawaters produced the lowest concentration of bacteria in comparison to seawater left open to air and that tested in the anaerobic chamber. Bubbling N₂ gas did not promote SRB growth.

pH values were recorded in KW seawater bubbled with mixed gases containing CO₂ ranging from

0 to 0.1%. Figure 4 shows pH values as a function of CO₂%. Increase in CO₂% resulted in decreased pH values. The highest pH value (8.74) was recorded with the 0% CO₂, and the lowest (7.73) with the 0.1% CO₂ gas mixture.

DISCUSSION

It is generally recognized that seawater surrogates do not approximate the complexity of natural seawater. However, many of the classic papers dealing with SRB corrosion in the marine environment have been conducted in artificial media to which nutrients and microorganisms have been added.^{10,16} One motivation for using synthetic seawater instead of natural seawater in MIC experiments is the goal of getting reproducible results. The dilemma is whether to start with a well-characterized controlled medium that may not replicate any natural environment or with a natural environment that cannot be thoroughly characterized and that varies with time. Few investigators have considered the differences that can be induced in natural seawater and synthetic seawater maintained under different experimental conditions. Dexter concluded that changes in pH in natural and synthetic seawater influenced the corrosivity of both toward aluminum.⁶

In the experiments described in this paper, sulfide production in two natural coastal seawaters was evaluated under two laboratory conditions: purging with nitrogen and maintenance in an anaerobic hood with an atmosphere of 10% H₂, 5% CO₂, and 85% N₂. It was demonstrated that the microbial population that one is able to detect is determined by the way the water is maintained. The microbial populations were followed using liquid culture techniques that are recognized as capable of growing only a small percentage of the natural populations. Liquid culture techniques can be used to make comparisons, but not to make determinations about population diversity. In the experiments described in this paper, bubbling N₂ into natural seawater produced a pH shift from 8.0 to above 9.0. Maintenance of seawater in an anaerobic hood with an anaerobic mixture of gases produced a pH shift of 8.0 to below 7.0. Using Figure 4, a gas mixture with 0.05% CO₂ will produce a seawater pH of 8.0, matching the measured pH values in the aerobic exposures (KW-A/PG-A) (Figure 1).

The reasons for higher SRB in KW seawater compared to PG water have not been explored. While evaluating the corrosion of aluminum, Dexter reported results that were internally consistent within a single batch of natural coastal seawater from a variety of locations, but were inconsistent from batch to batch at the same site and between sites.⁶

The point of this short technical note is not to question the conclusions of past research involving SRB in natural or synthetic seawater media purged with nitrogen without regard to potential changes in

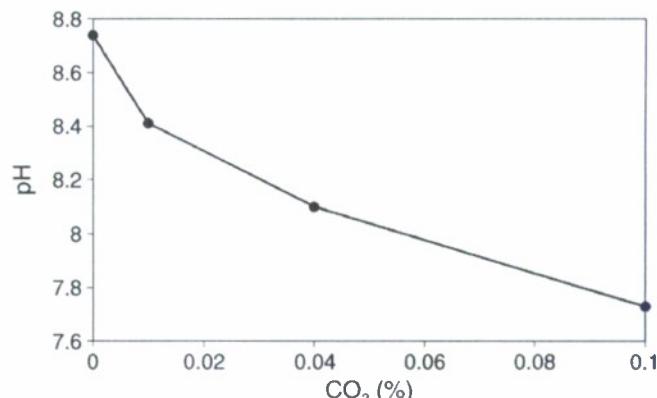


FIGURE 4. KW seawater pH as a function of CO₂% in bubbled mixed gas containing 10% H₂ and balance N₂.

chemistry and microflora. It is the intention of the authors to alert present and future researchers to the possibility that deaeration using pure nitrogen may influence microbial sulfide production. Currently, the authors use an anaerobic atmosphere consisting of 0.1% CO₂, 10% H₂, and balance N₂, which maintains seawater under anaerobic conditions with a pH of 7.8.

CONCLUSIONS

- ❖ Methods for deaerating natural coastal seawaters influenced the chemistry and microflora, particularly over time.
- ❖ Laboratory experiments that created anaerobic conditions by bubbling pure nitrogen gas into natural seawater produced pH changes that were not conducive to the growth of SRB.
- ❖ KW seawater consistently had equal or greater bacterial numbers in all conditions when compared with PG seawater.
- ❖ Despite the higher initial dissolved sulfate concentration in PG water, higher DS concentrations were measured in KW seawater.
- ❖ An anaerobic atmosphere consisting of 0.1% CO₂, 10% H₂, and balance N₂ can maintain seawater under anaerobic conditions at a pH of 7.8.

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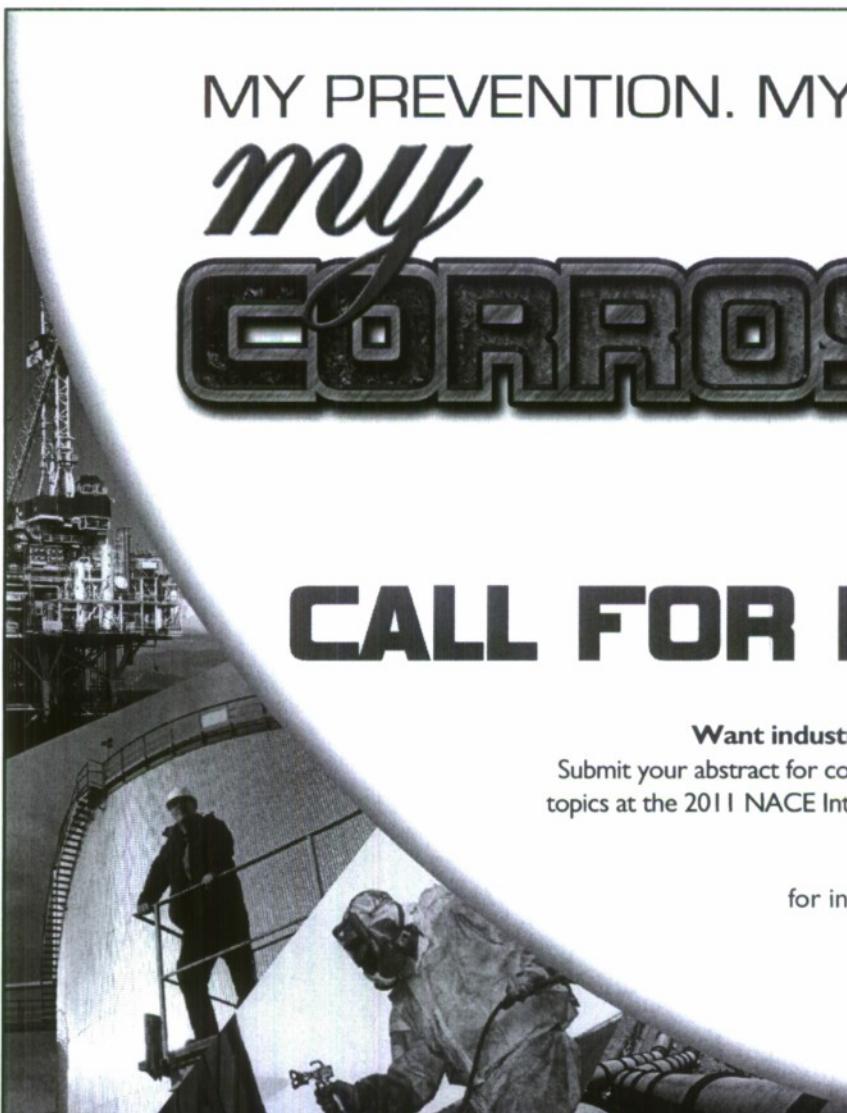
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